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The Human Papilloma Virus–16 E7 Oncoprotein Is Able to Bind to the Retinoblastoma Gene Product

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Deletions or mutations of the retinoblastoma gene, RB1, are common features of many tumors and tumor cell lines. Recently, the RB1 gene product, p105-RB, has been shown to form stable protein/protein complexes with the oncoproteins of two DNA tumor viruses, the adenovirus E1A proteins and the simian virus 40 (SV40) large T antigen. Neither of these viruses is thought to be associated with human cancer, but they can cause tumors in rodents. Binding between the RB anti-oncoprotein and the adenovirus or SV40 oncoprotein can be recapitulated in vitro with coimmunoprecipitation mixing assays. These assays have been used to demonstrate that the E7 oncoprotein of the human papilloma virus type–16 can form similar complexes with p105-RB. Human papilloma virus–16 is found associated with approximately 50 percent of cervical carcinomas. These results suggest that these three DNA viruses may utilize similar mechanisms in transformation and implicate RB binding as a possible step in human papilloma virus–associated carcinogenesis.

THE RETINOBLASTOMA GENE, RB1, is deleted or mutated in many human tumors or tumor cell lines, including retinoblastomas (*1, 2*), osteosarcomas (*1, 3*), small cell lung carcinomas (*4*), breast cancers (*5*), and bladder carcinomas (*6*). Inheriting a mutant allele for the RB1 gene predisposes a recipient to retinoblastoma (*7*). In all cases studied to date, the loss of the RB1 gene or the inability to synthesize p105-RB is correlated with increased cell proliferation and oncogenesis. These results have led to the hypothesis that the RB1 polypeptide plays a critical role in limiting the proliferation of certain cells. Loss of the RB protein would remove this block, thus indirectly stimulating cell proliferation.

In cells transformed or infected with adenovirus or SV40, the transforming proteins of these viruses form protein complexes with p105-RB (*8, 9*). It has been speculated that the binding of these oncoproteins to p105-RB inactivates the RB protein, thus mimicking the loss of the RB1 gene as seen in genetic predisposition to retinoblastoma. Neither SV40 nor adenovirus has been linked to human cancer (*10*). To test the possible link between the binding of p105-RB to other viral transforming proteins in the genesis of human cancer, an in vitro mixing assay (*11*) has been used to study the association of the E7 transforming protein of human papilloma virus–16 with p105-RB.

Papilloma viruses are often associated with benign, proliferative, squamous-epithelial lesions in higher vertebrates. In some cases these viruses have been associated with lesions that may progress to carcinomas. Compelling clinical and epidemiological data now link certain of the human papilloma viruses (HPVs) to a variety of human cancers, most notably cervical cancer. The most thoroughly studied of the HPVs associated with cervical carcinomas is HPV-16, the DNA of which has been found in over 50% of the cervical biopsy and tumor specimens examined (*12*). Studies with HPV-16 have revealed that the E7 gene is both sufficient and necessary for transformation of established rodent cells (*13, 14*). Furthermore, the E7 proteins from HPV-16 can cooperate with an activated *ras* oncogene to transform primary baby rat kidney cells in vitro (*14, 15*).

The transforming ability of the HPV-16 E7 protein is one of the characteristics shared with the early region 1A (E1A) proteins of adenovirus. In addition to their similar transforming abilities, E7 and E1A share transcriptional modulatory functions, in that both can stimulate transcription from the adenovirus E2 promoter (*14, 16*). Comparisons of the amino acid sequences of the E7 and E1A proteins have revealed marked similarities between the NH_2-terminus of E7 and portions of conserved regions 1 and 2 of E1A (Fig. 1) (*14, 17*). Conserved regions 1 and 2 of E1A have been shown to

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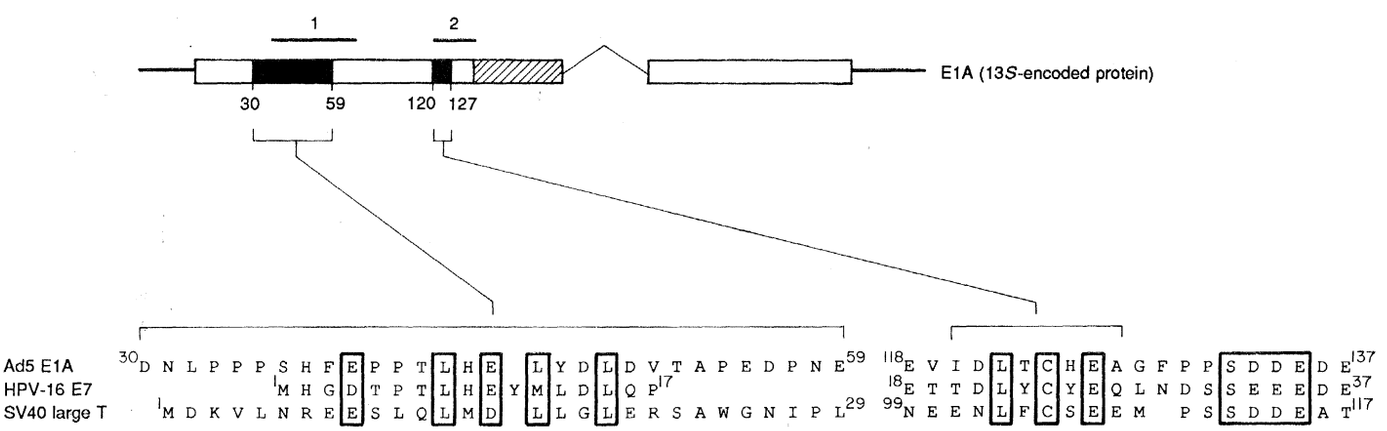

Fig. 1. Sequence homologies between the HPV-16 E7 and adenovirus E1A proteins. Regions of the E1A proteins known to be required for binding to p105-RB (*20*) are compared to homologous regions in the HPV-16 E7 proteins and the SV40 large T antigen. Only regions that are related to the p105-RB binding sites on E1A are listed. Other regions of homology are not shown [see (*14*) for further amino acid homologies]. Black boxes represent the minimal regions needed for binding of E1A to p105-RB. Corresponding regions in E7 and large T antigen are boxed if they are identical or if they represent conservative changes. Conserved regions 1 and 2 of E1A are indicated (*17*).

be important for such activities as cooperation with *ras*, stimulation of DNA synthesis, and the ability to repress transcriptional enhancement (*18, 19*). The structural and functional similarities between these two oncoproteins suggest that they may function through similar mechanisms.

Recently, the minimal regions of E1A needed for transformation have been defined (*19*). These regions correspond precisely to the binding sites for three cellular proteins, suggesting that these proteins are the targets for E1A-mediated transformation (*20*). One of these cellular proteins has recently been identified as the protein product of the retinoblastoma (RB) gene, known as p105-RB (*8*). The binding sites for p105-RB include a portion of conserved regions 1 and 2, suggesting that the E7 protein might also bind to p105-RB. In support of this supposition is the recent finding that the large T antigen of SV40 binds p105-RB and that large T antigen contains sequences homologous to the p105-RB binding sites on E1A (*9, 21*).

The in vivo interaction between adenovirus E1A and p105-RB can be reproduced in vitro by mixing E1A-containing cell lysates with RB polypeptides (Fig. 2). In this assay, radioactively labeled RB polypeptides were synthesized in rabbit reticulocyte lysates programmed with RNA prepared by in vitro transcription from the RB cDNA (*8*). No full-length RB polypeptides were synthesized in this system for reasons that are not clear at present. However, these translation reactions yielded a nested set of overlapping polypeptides that resulted from initiation of translation at internal AUG codons and therefore share a common COOH-terminus (*8*). This is demonstrated in Fig. 2 where all of the major products of the in vitro translation were immunoprecipitated directly by antibodies raised against a peptide corresponding to the COOH-terminal 15 amino acids of the deduced RB protein sequence (compare the +RB RNA lane with the αRB 144 lane). In contrast, the monoclonal antibody C36, which recognizes an epitope in the NH_2-terminal half of the RB protein, immunoprecipitated only a subset of these proteins. When the RB polypeptides were mixed with cell lysates containing E1A, a characteristic subset of the RB polypeptides bound to the E1A proteins and could be coimmunoprecipitated via a monoclonal antibody to E1A. When labeled RB polypeptides were mixed with lysates that did not contain E1A or were immunoprecipitated with control antibodies, no precipitation of labeled RB polypeptides occurred. Similar patterns were detected when purified SV40 large T antigen was used in place of the E1A-containing lysates in analogous mixing reactions (*22*).

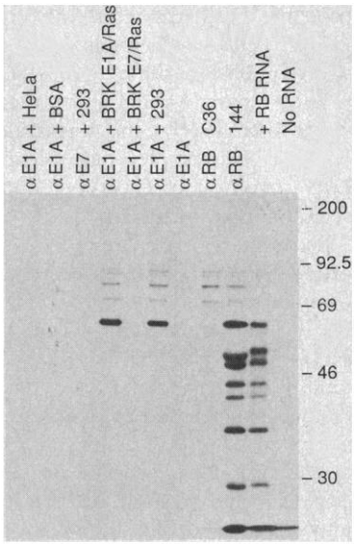

Fig. 2. Coimmunoprecipitation of RB polypeptides after mixing with adenovirus E1A–containing cell lysates. The conditions used for these mixing experiments have been described elsewhere (*11*), but are briefly described below. [^{35}S]Methionine-labeled polypeptides were synthesized by in vitro transcription/translation of the human RB cDNA (*8*) and mixed with detergent lysates of human 293 cells (18 μg of total protein), HeLa cells (0.69 mg of total protein), or baby rat kidney cells (BRK) cotransfected with an activated *ras* gene and either E1A or HPV-16 E7 (1.17 and 1.33 mg of total protein, respectively). Control tubes were diluted with equivalent volume (200 μl) of lysis buffer (250 m*M* NaCl, 0.1% NP-40, and 50 m*M* Hepes, *p*H 7.0) with or without 5 mg of bovine serum albumin (BSA) per milliliter. Mixes were incubated 90 min on ice, then immunoprecipitated with M73, a monoclonal antibody specific for E1A protein (*28*), C36, a monoclonal antibody specific for p105-RB (*8*), αE7, a polyclonal antibody raised against an HPV-16 E7-trp fusion protein (*24*), or 144, a rabbit antisera raised against a synthetic peptide corresponding to the COOH-terminal 15 amino acids of the human RB protein (*8*). The immunoprecipitated proteins and the translation products from reactions containing RB cRNA (2.5 μl of the total reaction) were resolved on a 10% polyacrylamide gel (*29*) and detected by fluorography (*30*). Numerical markers are in kilodaltons.

Since translation of the RB cRNA produced a series of overlapping polypeptides from initiation at internal AUG codons, the precipitation patterns could be used to map the approximate region of p105-RB needed to bind to E1A. The smallest polypeptide coprecipitated via E1A antigen migrated at approximately 60 kD (Fig. 2), and this locates the binding site on the retinoblastoma protein to the COOH-terminal 541 amino acids.

We used the in vitro binding assay described above to determine whether the HPV-16 E7 protein could also bind to RB polypeptides. Cell lysates were prepared from CaSki cells, a human cervical carcinoma cell line that contains HPV-16 DNA (*23*) and expresses HPV-16 E7 protein (*24, 25*), mixed with labeled RB polypeptides, and immunoprecipitated by polyclonal antibodies to E7 (Fig. 3A). The pattern of RB polypeptides bound to E7 was identical to that seen with adenovirus E1A and SV40 large T antigen, showing that the E7 proteins were capable of binding to the RB protein and further suggesting that a similar region of p105-RB is required for binding in each case.

In a reciprocal experiment (Fig. 3B), a clone containing the HPV-16 E7 open reading frame was transcribed and the cRNA translated to produce labeled E7 protein. This lysate was treated with C36, a monoclonal antibody to p105-RB (*8*), to remove p105-RB, and then mixed with a cell lysate prepared from SK-NMC or NGP cells, two neuroblastoma cell lines that contain normal p105-RB protein. From this mix, labeled E7 could be coprecipitated with C36, although E7 was not recognized directly by the antibody itself, indicating that E7 and p105-RB had associated. The levels of bound E7 protein varied approximately tenfold in this experiment, which may reflect different levels of active p105-RB protein found in these cells.

Finally, mixing of in vitro–translated E7 with in vitro–translated retinoblastoma polypeptides enables labeled E7 to be coimmunoprecipitated by an antibody to p105-RB and labeled RB polypeptides to be coimmunoprecipitated by polyclonal antibodies specific for E7 (Fig. 3C). In this experiment the degree of coprecipitation was low, presumably because of the low concentration of the two labeled proteins. In the experiments described earlier, we assume that the cold protein was introduced in a molar excess, thus facilitating the binding.

These results demonstrate that coimmunoprecipitation of HPV-16 E7 and p105-RB occurs after mixing in vitro. From this we conclude that E7 and p105-RB are capable of complex formation. This raises several questions concerning the HPV interactions with RB. First, do analogous complexes occur in vivo? We have analyzed cervical carcinoma cell lines for the presence of an E7/p105-RB complex. These experiments are technically difficult because of the low levels of the E7 proteins, and in preliminary experiments we have failed to show coimmunoprecipitation of these proteins in two HPV-16 E7-expressing cell lines [CaSki, SiHa (*23, 24*)]. The reasons for this anomaly are not clear. It is possible that only a small proportion of p105-RB or E7 or both in cells is involved in complex formation and therefore is difficult to detect. Such a situation might result from cell cycle regulation or modification of either of these

proteins. Furthermore, if binding by the viral proteins causes an allosteric modification of p105-RB, the E7/p105-RB complex may be rapidly formed and turned over in vivo. Alternatively, the formation of the E7/p105-RB complex may occur early in HPV infection and be lost during later stages of carcinogenesis.

Second, at what stage of HPV-infection or HPV-associated carcinogenesis could the complex formation between E7 and p105-RB be important? The existing evidence suggests that specific HPVs have a necessary but not sufficient role in associated anogenital malignancies, and the part played by E7 is unclear. E7 sequences are conserved in the anogenital HPVs regardless of the associated risks for progression, and it is of primary importance to determine whether E7 proteins from all HPV types can bind to p105-RB. These experiments are currently under way. Part of the solution to this problem may lie in the regulation of E7 expression by these different viruses. In cervical carcinomas associated with HPV-16, -18, and -33, the viral DNA is usually integrated and transcriptionally active (*23, 24, 26*). In those cancers and derived cell lines that have been closely examined, integration has often occurred in the E1/E2 region, resulting in deregulated expression of the E6 and E7 genes (*27*). It is possible that the presence of a deregulated E7 protein and its subsequent ability to complex with p105-RB may be a critical feature in the multistep tumorigenesis.

The observation that the transforming proteins of three different types of DNA tumor viruses are able to bind the product of the retinoblastoma gene implicates the binding of RB as a common mechanism in transformation by these proteins. This lends support to the emerging view that the RB protein plays a key role in the control of cell proliferation, and adds another example of how oncogenes and anti-oncogenes may confront one another in the same regulatory network.

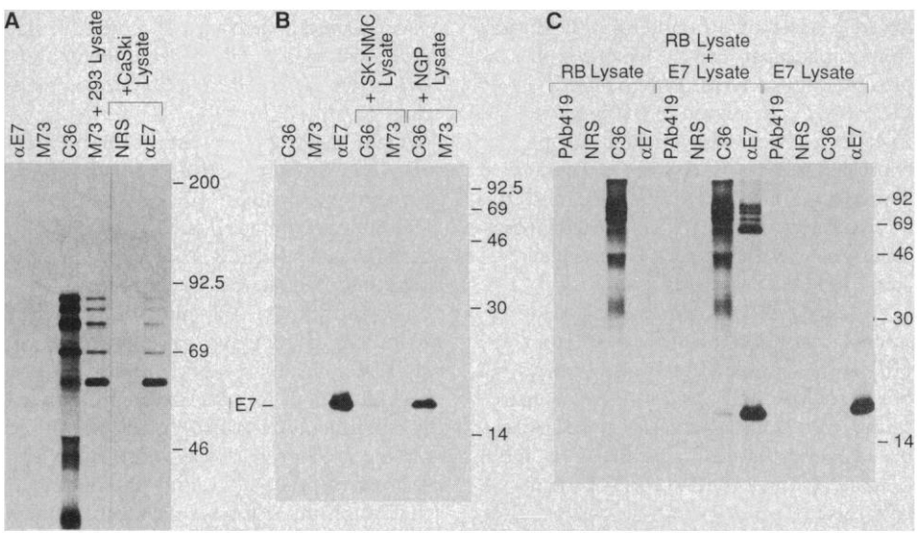

Fig. 3. Coimmunoprecipitation of RB polypeptides and E7 polypeptides after mixing in vitro. Assays were performed as described in Fig. 2. (**A**) Assays with labeled RB polypeptides. ^{35}S-labeled RB polypeptides were synthesized and mixed with detergent lysates from human 293 cells (18 μg of protein) or CaSki cells (0.38 mg of protein). Proteins were immunoprecipitated with M73, a monoclonal antibody specific for E1A, C36, a monoclonal antibody specific for p105-RB, αE7 polyclonal antibodies, or normal rabbit serum (NRS). Immunoprecipitated proteins were separated on an 8% polyacrylamide gel and visualized by fluorography. (**B**) Assays with labeled E7 polypeptides. [^{35}S]Methionine-labeled E7 polypeptides were synthesized by in vitro transcription/translation of the HPV-16 E7 gene. The E7 open reading frame was cloned downstream of the T7 promoter in pGEM-1, and the plasmid was cleaved at the Pst I site downstream of the inserted sequences. Complementary RNAs were synthesized identically to the RB cRNA and used to program translation in rabbit reticulocyte lysates (1 μg of cRNA per 50-μl reaction). After translation the reticulocyte lysates were cleared with C36 to remove any p105-RB. Cleared E7 reticulocyte lysates were mixed (70 μl of reaction mix in 350 μl of lysis buffer) with 200 μl of lysates prepared from neuroblastoma cell lines SK-NMC and NGP (which contained 0.52 and 0.44 mg of total protein, respectively) or 200 μl of lysis buffer (for controls). After 90 min on ice, the solution was divided into aliquots, proteins were immunoprecipitated either with C36 or M73 monoclonal antibodies or with αE7 polyclonal antibodies, and the resulting proteins were separated on a 15% polyacrylamide gel. Labeled proteins were visualized by fluorography. (**C**) Mixing of in vitro–synthesized E7 and RB polypeptides. ^{35}S-labeled polypeptides were prepared and the E7 lysate cleared of p105-RB as described above and in Fig. 2. The two rabbit reticulocyte lysate reactions were diluted in lysis buffer and mixed to contain 70 μl RB:50 μl E7. After mixing for 3 hours, the solutions were divided into aliquots, and proteins were immunoprecipitated with either C36 monoclonal antibody or E7 polyclonal antibodies, and we used PAb419 (*31*) or normal rabbit serum (NRS) as negative controls. Labeled proteins were separated on a 15% polyacrylamide gel and detected by fluorography.

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Point Mutational Inactivation of the Retinoblastoma Antioncogene

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The retinoblastoma (Rb) antioncogene encodes a nuclear phosphoprotein, p105-Rb, that forms protein complexes with the adenovirus E1A and SV40 large T oncoproteins. A novel, aberrant Rb protein detected in J82 bladder carcinoma cells was not able to form a complex with E1A and was less stable than p105-Rb. By means of a rapid method for the detection of mutations in Rb mRNA, this defective Rb protein was observed to result from a single point mutation within a splice acceptor sequence in J82 genomic DNA. This mutation eliminates a single exon and 35 amino acids from its encoded protein product.

GENETIC ANALYSIS OF RETINOBLASTOMAS and osteosarcomas has revealed a cellular gene, *RB-1*, whose homozygous inactivation is a critical determinant of tumorigenesis (*1*). Because the absence of functional Rb alleles correlates with tumor formation, it is widely assumed that this gene acts to constrain the growth of normal cells. Its loss is thought to remove a barrier to cell proliferation, leading in turn to the growth of tumor cell clones.

Recently we and others reported isolation of a molecular clone of a gene that has many of the expected properties of the Rb gene (*2–4*). For example, structural analysis of chromosomal deletions found in a small number of retinoblastomas and osteosarcomas led to the conclusion that this cloned gene, and not neighboring genes, is the common target of the random chromosomal deletions that triggered these tumors. By means of Southern blot analysis, deletions of the Rb gene have been found in only 15 to 40% of retinoblastoma tumors examined to date (*2–5*). It has been assumed that the Rb gene may be altered in some or all of the remaining tumors in subtle ways that are not detectable by Southern blotting but, nevertheless, suffice to inactivate the function of the Rb gene. We report here just such a mutation, discovered through analysis of the Rb-encoded proteins found in a number of human tumor cell lines.

The Rb gene encodes a nuclear phosphoprotein of approximately 105 kD, p105-Rb, that is associated with DNA-binding activity (*6, 7*). Recent experiments have suggested that this protein may also be involved in alternative tumorigenic mechanisms that do not depend upon mutational alteration in the Rb gene itself. Specifically, we have found that in adenovirus-transformed cells, the virus-encoded E1A oncogene proteins are complexed with as many as ten host cell proteins. Among these is a protein of 105 kD that we have identified as p105-Rb (*7*). Detailed studies of E1A mutants have established that mutations disrupting the binding of E1A to p105-Rb ablate the transforming powers of the E1A oncoprotein (*8, 9*). We concluded that the same protein (p105-Rb) found to be absent from spontaneously arising human retinoblastomas is also perturbed by E1A proteins as part of adenovirus-mediated transformation. Evidence from one of our laboratories as well as others has established that p105-Rb is a common target for protein binding by two other oncoproteins, SV40 large T antigen and the E7 protein of human papillomavirus (*10, 11*).

We have used the formation of the E1A:p105-Rb protein complex as an assay for the presence and function of p105-Rb. To this end, normal and tumor cell lines were infected with human adenovirus type 5. Any resulting E1A:p105-Rb complexes were detected in these cells via immunoprecipitation with a monoclonal antibody, M73 (*12, 13*), that reacts specifically with the E1A oncoproteins. Such a test had shown the presence of E1A:p105-Rb complexes in most infected tumor cell types and its absence from several adenovirus-infected retinoblastoma cell lines (*7*). This absence was in consonance with the loss of functional p105-Rb from retinoblastomas. Unexpectedly, we also were unable to find E1A:p105-Rb complexes in adenovirus-infected human J82 bladder carcinoma cells (Fig. 1A). Changes in the Rb gene with loss of p105-Rb have not previously been associated with bladder carcinomas.

Use of a monoclonal antibody, C36, specific for the Rb protein (*7*) and two independent rabbit anti-peptide sera reactive with the Rb protein (*7*) made it possible to determine whether this protein was present in the cells of the J82 tumor line. Immunoprecipitations with C36 monoclonal antibody (Fig. 1A), antiserum prepared against an internal synthetic peptide, #147, or antiserum #144 (which reacts with the COOH-terminus) all precipitated an Rb-related protein of approximately 102 kD (Fig. 1B). The C36 monoclonal antibody also precip-

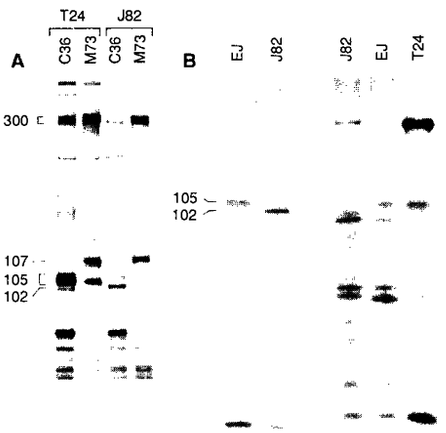

Fig. 1. Immunoprecipitation of Rb protein and E1A:Rb protein complexes from bladder carcinoma cells. (**A**) Cultures of J82 and T24 bladder carcinoma cells were infected with adenovirus type 5 and 10 hours later incubated for 3 hours with [^{35}S]methionine. Cell lysates were prepared as described (*7*) and immunoprecipitated with monoclonal antibodies against Rb protein (C36)(*7*) or adenovirus E1A proteins (M73) (*12*). Immunoprecipitates were resolved by electrophoresis for 2.5 hours on an 8%–4% discontinuous SDS-polyacrylamide gel, processed for fluorography, and exposed for 2 days at −70°C. Indicated are the sizes of the Rb proteins precipitated from J82 and T24 cells, as well as two cellular proteins, 300 kD and 107 kD, that are coprecipitated by M73. (**B**) Cultures of J82, EJ, and T24 bladder carcinoma cells were incubated with [^{35}S]methionine and cell lysates prepared as in (A). Immunoprecipitations of Rb protein were performed with two independently derived rabbit sera to the synthetic peptides characterized previously (*7*). Rabbit sera #147 (reactive with an epitope internal to the Rb protein) was utilized in the two leftmost lanes, and rabbit sera #144 (reactive with the Rb COOH-terminus) in the rightmost three lanes.

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